2010

Prion genotypes of scrapie-infected Canadian sheep 1998–2008

Noel P. Harrington
Canadian Food Inspection Agency

Katherine I. O'Rourke
U.S. Department of Agriculture, katherine.orourke@ars.usda.gov

Yuquin Feng
Canadian Food Inspection Agency

Jasmine Rendulich
Canadian Food Inspection Agency

Cathleen DiFruscio
Canadian Food Inspection Agency

See next page for additional authors

Prion genotypes of scrapie-infected Canadian sheep 1998–2008

Noel P. Harrington, Katherine I. O'Rourke, Yuqin Feng, Jasmine Rendulich, Cathleen DiFruscio, Aru Balachandran

Abstract

This report describes the genetics of the prion protein gene *(PRNP)* at codons 136, 154, and 171 for sheep diagnosed with naturally acquired classical scrapie in Canada between 1998 and 2008. Genotyping analysis was performed on 249 sheep with confirmed classical scrapie infection representing 98 flocks from 6 provinces. A further case-control analysis of 3 of these flocks compared the genotypes between infected sheep ($n = 72$) and those of their healthy flockmates ($n = 1990$). The incidence of classical scrapie in the Canadian sheep population was highly associated with the ARQ haplotype (91.8%) and the ARQ/ARQ genotype (91.6%). In addition, the ARQ haplotype was found at significantly higher frequency in scrapie-infected sheep when compared with their healthy flockmates. Comparison with other published data suggests that the scrapie risk of *PRNP* genotypes differs between Canada and countries where the VRQ allele is associated with the highest susceptibility to infection.

Résumé

Cet article décrit la génétique du gène de la protéine prion (PRNP) *aux codons 136, 154, et 171 pour des moutons avec un diagnostic de tremblante acquise naturellement au Canada entre 1998 et 2008. L'analyse génotypique a été effectuée sur 249 moutons avec un diagnostic confirmé de tremblante classique représentant 98 troupeaux dans 6 provinces. Une analyse cas-témoin ultérieure de 3 de ces troupeaux a comparé les génotypes entre des moutons infectés* (n = *72) et des congénères en santé* (n = *1990). L'incidence de tremblante classique dans la population ovine canadienne était fortement associée avec l'haplotype ARQ (91,8 %) et le génotype ARQ/ARQ (91,6 %). De plus, l'haplotype ARQ a été retrouvé à des fréquences significativement plus élevées chez les moutons infectés par la tremblante comparativement à leurs congénères en santé. Une comparaison avec les autres données publiées suggère que le risque de tremblante pour les génotypes* PRNP *diffère entre le Canada et les pays où l'allèle VRQ est associé avec la susceptibilité la plus élevée à l'infection.*

(Traduit par Docteur Serge Messier)

Scrapie is a naturally occurring fatal neurodegenerative disease of sheep and goats that belongs to the group of prion diseases known as transmissible spongiform encephalopathies (TSEs), which also includes bovine spongiform encephalopathy (BSE) of cattle and chronic wasting disease (CWD) of elk and deer. These complex diseases are characterized by long incubation periods, disturbances in behavior and movement, degeneration of tissues of the central nervous system (CNS), and accumulation of a disease-causing isoform of the host-encoded cellular prion protein (PrP). Clinical cases of scrapie in small ruminants have been recorded for many decades in many regions of the world; notable exceptions are Australia and New Zealand. The first report of scrapie in Canada dates back to 1938, and scrapie was subsequently made a federally reportable disease with the establishment of control programs as early as 1945. The disease, however, has proven to be notoriously difficult to control and can persist within a flock by horizontal transmission between flockmates and via vertical transmission from ewe to lamb (1). Since 1984, there have been 167 flocks in 6 Canadian provinces with confirmed cases of classical scrapie (2). Flocks infected with scrapie may experience significant production losses, but scientific study of the disease was limited until the 1990s when interest was triggered by the BSE epidemic in British cattle and its connection to variant Creutzfeld-Jakob disease (vCJD) in humans. Although scrapie is not transmissible to humans, the possibility that BSE may circulate in small ruminant populations made the TSEs of sheep and goats a food safety concern as well as an animal health issue (3).

The ineffectiveness of conventional infectious disease control measures against scrapie has prompted the adoption of innovative strategies. Specific single nucleotide polymorphisms within the protein-coding sequence of the prion gene *(PRNP)*, and the resulting amino acid changes, have been associated with scrapie susceptibility and disease progression [reviewed in (4)]. The 3 most studied codons

National Reference Laboratory for Scrapie and CWD, Ottawa Laboratory Fallowfield, Ottawa, Canadian Food Inspection Agency, Ottawa, Ontario (Harrington, Feng, Rendulich, DiFruscio, Balachandran); Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, Washington, USA (O'Rourke).

Address all correspondence to Dr. Noel Harrington; telephone: (613) 228-6698; fax: (613) 228-6669; e-mail: noel.harrington@inspection.gc.ca

Dr. Harrington's current address is Ottawa Laboratory Fallowfield, Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, Ontario K2H 8P9.

Received June 4, 2009. Accepted September 24, 2009.

Table I. Primer and probe sequences used in the dual fluorescent multiprobe allelic discrimination assay

Primer/ Probe name	Sequence (5′ → 3′)	Amino acid
Forward primer	GCCTTGGTGGCTACATG	
Reverse primer	CTGTGATGTTGACACAGTCAT	
A136-probe	*FAM*-CTGCTCATGGCACTTCCCAG-*BHQ1*	Ala
V136-probe	*HEX*-CTGCTCATGACACTTCCCAG-*BHQ1*	Val
R154-probe	*TexasRed*-CCGTTACTATCGTGAAAACATGTAC-*BHQ2*	Arg
H154-probe	*Cy5*-CCGTTACTATCATGAAAACATGTACC-*BHQ2*	His
R171-probe	*FAM*-CCAGTGGATCGGTATAGTAACCA-*BHQ2*	Arg
H171-probe	*HEX*-AGACCAGTGGATCATTATAGTAACCA-*BHQ1*	His
Q171-probe	*TexasRed*-CCAGTGGATCAGTATAGTAACCAGA-*BHQ2*	Glu
K171-probe	*Cy5*-CCAGTGGATCAATATAGTAACCAGA-*BHQ2*	Lys

Fluorescent labels and quenchers are in italics; polymorphisms are underlined.

associated with scrapie susceptibility in sheep are those encoding amino acid residues at positions 136 (A/V), 154 (R/H), and 171 (Q/R/H/K) (where alternative amino acid alleles are indicated by their single letter codes). Combinations of polymorphisms at these codons give rise to 5 commonly seen *PRNP* codon haplotypes or alleles: $A_{136}R_{154}R_{171}$ (ARR) and, continuing this notation, AHQ, ARH, VRQ, and ARQ. These 5 alleles can be arranged to give 15 *PRNP* diploid genotype combinations commonly found in sheep (such as, ARR/ARR, VRQ/ARQ, ARH/AHQ).

The susceptibility of sheep to scrapie has been shown to differ widely between genotypes, ranging from greatest resistance for the ARR/ARR genotype to extreme susceptibility for the VRQ/VRQ genotype (5). Such information has been used to assign genotypes to different classes of susceptibility for the purpose of ease of interpretation and facilitating the design of national genotype-based scrapie control and eradication programs such as the National Scrapie Plan (NSP) of Great Britain and equivalent plans in other countries.

The association between specific *PRNP* genotypes and susceptibility to classical scrapie has not previously been reported for Canadian sheep. The objectives of this study were, first to describe the *PRNP* gene polymorphisms at codons 136, 154, and 171 for sheep diagnosed with classical scrapie in Canada between 1998 and 2008, and second, to conduct analysis of 3 fully genotyped scrapie-affected flocks comparing the *PRNP* polymorphisms of the scrapie-affected animals with those of their healthy flockmates.

A total of 284 sheep from 98 flocks confirmed as scrapie-infected as part of the Canadian Food Inspection Agency (CFIA) national scrapie surveillance and control program between 1998 and 2008 were examined. Case-control analysis involved the genotyping of all sheep (healthy and scrapie-infected) from 3 of these flocks identified in 2008 from different geographical regions. Selective culling was applied in the scrapie-affected flocks such that sheep carrying susceptible genotypes were culled and tested for scrapie infection while those with resistant or less susceptible *PRNP* genotypes were kept alive. The number of scrapie-affected sheep genotyped was 72 (n = 4, 4, and 64 from flocks A, B, and C, respectively) and the number of healthy flockmates genotyped was 1990 (n = 74 549, and 1367 from flocks A, B, and C, respectively).

The source of tissue for DNA extraction was either whole blood (200 μL) collected into EDTA-vacutainer tubes (Becton Dickinson, Oakville, Ontario), frozen archived brain tissue (30 mg), or archival paraffin-embedded formalin-fixed brain tissue (two 5-μm thick sections). High molecular weight genomic DNA was extracted using the appropriate tissue kit (MagNa Pure DNA Isolation Kit I for blood and MagNa Pure DNA Isolation Kit II for brain and paraffin-embedded tissue; Roche Applied Sciences, Laval, Quebec) following the manufacturer's instructions. Real-time polymerase chain reaction (qPCR) amplification of a 180 base pair (bp) DNA fragment from the *PRNP* gene exon 3 (GenBank accession number M31313) that included codons 136, 154, and 171 was performed as described by Van Poucke et al (6) with minor modifications as described below. Allelic variants at each codon were identified with 1 of 8 allele-specific dual-labelled probes (Integrated DNA Technologies, Coralville, Iowa, USA) and are listed in Table I. Two independent real-time PCR reactions were employed: one for codons 136 and 154 and one for codon 171. In PCR 1, the 4 allelic variants of codons 136 (A/V) and 154 (R/H) were determined whereas PCR 2 determined the 4 allelic variants of codon 171 (R/H/Q/K). Both PCRs of the assay were performed in the iCycler iQ5 Real-time PCR Detection System (Bio-Rad) using 96-well PCR plates (Bio-Rad) in a total volume of 15 μL containing iQ Supermix (50 mM KCl, 20 mM Tris-HCl pH 8.4, 0.8 mM dNTPs, 0.375 U iTaq DNA polymerase, 3 mM $MgCl_2$ and stabilizers; Bio-Rad), 400 nM of each primer and probe, and genomic DNA (150–200 ng). Each sample was tested in duplicate, and appropriate negative controls and positive controls from animals of known genotype were included in each run (kindly provided by Michael Heaton, United States Department of Agriculture). The thermocycling protocol involved an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation for 20 s at 95°C and annealing-elongation for 40 s at 62°C. Fluorescence was measured for each cycle at the end of the annealing step and data was analyzed to assign allele reactivity using iQ5 Optical System Software (Bio-Rad).

The association between scrapie status and haplotype for the case-control study was analyzed statistically using the chi-squared test for independence or the Fisher's exact test (MedCalc Software Version 8.1.0.0, Mariakerke, Belgium).

Table II. The *PRNP* haplotype and genotype frequencies of 249 sheep confirmed as scrapie-infected in Canada between 1998 and 2008

	Number	%
Haplotypes		
ARR	3	0.6
AHQ	2	0.4
ARQ	457	91.8
ARH	13	2.6
VRQ	23	4.6
Genotypes		
ARQ/AHQ	1	0.4
ARQ/ARQ	228	91.6
VRQ/ARR	3	1.2
VRQ/AHQ	1	0.4
VRQ/ARQ	13	5.2
VRQ/VRQ	3	1.2

Sheep confirmed as scrapie-infected were between 2 and 8 years of age from 98 flocks located in 6 provinces (Quebec, $n = 63$; Manitoba, $n = 15$; Ontario, $n = 9$; Saskatchewan, $n = 9$; Alberta, $n = 1$; and New Brunswick, $n = 1$). The majority of scrapie-affected sheep ($n = 216$) were crossbreds. The remainder consisted of purebreds including Suffolk ($n = 14$), Romanov ($n = 8$), Rideau Arcott ($n = 2$), Hampshire ($n = 1$), and sheep ($n = 43$) for which breed information was unavailable. Depending on tissue availability, DNA was extracted either from blood, archival frozen brain tissue, or archival paraffin-embedded formalin fixed brain tissue. The DNA of 35 animals was of insufficient quantity or quality for subsequent genotype analysis. The *PRNP* allelic frequencies of the remaining 249 Canadian scrapie-positive sheep are summarized in Table II. All 5 possible *PRNP* allelic variants associated with susceptibility or resistance to scrapie were detected: ARR, ARQ, ARH, AHQ, and VRQ. The scrapie-infected sheep, however, showed low genotype variability, with only 6 of the 15 commonly reported *PRNP* genotypes (ARQ/ARQ, ARQ/AHQ, VRQ/ARR, VRQ/AHQ, VRQ/ARQ, and VRQ/VRQ). Of the scrapie-infected sheep, the ARQ variant was the dominant allele with a haplotypic frequency of 91.8% and, similarly, ARQ/ARQ was the dominant genotype representing 91.6% of cases. Genotypes containing the ARR-resistant haplotype and either ARR, AHQ, ARQ, or ARH were not present in any scrapie-infected sheep. In particular, the ARR/ARQ genotype, which is one of the most common sheep genotypes in Canada (7), did not have any reported cases of classical scrapie in this study. The exception with respect to the ARR allele was the genotype VRQ/ARR which was observed in 3 (1.2%) scrapie-infected sheep. Notably, the reportedly high susceptibility genotype, VRQ/VRQ, was present in only 3 (1.2%) of Canadian scrapie-infected sheep. Due to the small number of purebred scrapie-infected sheep, and in some cases insufficient breed data, associations between breed, genotype, and scrapie susceptibility could not be examined in this study.

Although the large majority of Canadian scrapie cases were of the ARQ/ARQ genotype, fully quantifying the link between genotype and the risk of scrapie is difficult because it requires knowledge of the genotypes of the sheep that develop scrapie and those that do not. To that end, the genotypes of infected sheep were compared with their healthy flockmates for 3 recently identified and fully genotyped scrapie-affected flocks. Sheep from these flocks had all 5 possible *PRNP* allelic variants and 13 of the 15 common *PRNP* genotypes (Table III). The allele frequencies present in the 3 flocks ranged between 51.4% to 73.1% for the ARQ allele, 26.9% to 41.0% for the ARR allele, and 0% to 3.5% for the VRQ, AHQ, ARH, and ARQ alleles. The most frequent genotypes present in the case-control sheep were ARQ/ARR (39.9%) and ARQ/ARQ (40.5%).

Table III. Haplotype and genotype frequency (%) of healthy and scrapie-infected animals from 3 scrapie-affected flocks genotyped in 2008

	Healthy		Scrapie-infected	
	Number	%	Number	%
Haplotypes				
ARR	1320	33.2	0	0
AHQ	44	1.1	0	0
ARQ	2475	62.2	71	99.3
ARH	40	0.3	0	0
VRQ	129	3.2	1	0.7
Genotypes				
ARR/ARR	220	11.1	0	0
ARR/AHQ	12	0.6	0	0
ARR/ARH	3	0.2	0	0
ARR/ARQ	823	41.4	0	0
AHQ/AHQ	1	0.1	0	0
ARH/AHQ	1	0.1	0	0
AHQ/ARQ	28	1.4	0	0
ARH/ARH	0	0	0	0
ARH/ARQ	9	0.5	0	0
ARQ/ARQ	765	38.4	71	98.6
VRQ/ARR	42	2.1	0	0
VRQ/AHQ	1	0.1	0	0
VRQ/ARH	0	0	0	0
VRQ/ARQ	84	4.2	1	1.4
VRQ/VRQ	1	0.1	0	0

In addition to the index cases, several additional scrapie cases were identified amongst culled sheep. Overall, 72 of 2062 sheep form the 3 case-control flocks tested positive for classical scrapie. A comparison of the specific genotypes of healthy and scrapie-infected sheep shows that 11 genotypes occurred exclusively in the healthy sheep whereas only 2 genotypes were associated with both healthy and scrapie-infected sheep. The frequency of the ARQ/ARQ genotype in scrapie-infected animals (98.6%) was higher than in the healthy sheep (38.4%). Based on statistical analysis of these data of each flock using the χ^2 test, we concluded that there was a significant association between the ARQ haplotype and scrapie incidence ($P < 0.001$). In contrast, there was no significant association between the VRQ haplotype and scrapie incidence.

The potential for *PRNP* genotyping as a means to control classical scrapie emerged in the 1990s when case-control studies demonstrated a clear linkage between disease risk and *PRNP* genotype in several breeds of sheep in Britain. The ARR allele was associated with the highest level of protection in all breeds that carry it, and

seems to have a dominant influence so that heterozygotes as well as homozygotes are at low risk if exposed to the scrapie agent. On this basis, scrapie control and elimination strategies have been adopted, to a greater or lesser extent, by several European countries and in North America. This is the first report to describe the *PRNP* gene polymorphisms of scrapie-infected sheep in Canada. The susceptibility associated with the ARQ allele was eliminated in the presence of the ARR allele and, indeed, ARR/ARR and ARR/ARQ were the only numerically significant genotypes of Canadian sheep for which no scrapie cases were reported. These findings support, therefore, the notion that a breeding strategy that produces a national flock encoding the ARR allele at high frequency will be largely resistant to the classical scrapie stain currently circulating in Canada.

Although such programs have demonstrated that large-scale, industry-wide selection of *PRNP* genotype is feasible, they raise important issues including possible unintended impacts on performance traits, inbreeding, and genetic diversity in commercial populations. Although little or no consistent associations have been found between scrapie genotypes and the above traits (8), selection for genotypes resistant to classical scrapie may result in greater susceptibility to other scrapie strains. In particular, genotypes with increased resistance to classical scrapie, including ARR/ARR, have been found to be susceptible to the recently identified atypical strain (originally termed Nor98) of scrapie (9). The potential for unintended selection of a new strain of scrapie should caution regulatory authorities against any *PRNP* selection strategy that takes a sheep population towards homogeneity.

The findings of this study are significant in light of what was known regarding the VRQ allele and the nature of the scrapie agent(s) circulating in Canada. Generally classified as a high-risk factor for scrapie infection, the presence of healthy homozygote VRQ/VRQ sheep of old age in scrapie-affected sheep flocks is rare. It has been assumed that scrapie agent(s) that exist in Canada originated in the UK. However, although the Canadian and UK national sheep flocks have similar allelic frequencies for the 5 major alleles including VRQ (7,10), the *PRNP* genotype profile of scrapie-infected sheep contrasts sharply between the 2 nations. In the UK approximately 64% of the scrapie-infected sheep are of the VRQ/VRQ genotype, whereas only 1.2% of scrapie-infected Canadian sheep of this study were of this genotype. Conversely, most of the scrapie-infected sheep in Canada were of the ARQ/ARQ genotype (92%), whereas only 8% of scrapie-infected UK sheep are of this genotype. One possible explanation for such contrasting results is the possibility that the Canadian scrapie-affected flocks consisted of so-called non-VRQ breeds (with no or only occasional VRQ carriers). This hypothesis is inconsistent, however, with the frequency of the VRQ haplotype present in the general Canadian sheep population (~7%) (7) from which the scrapie-infected sheep originated and given that the majority of Canadian scrapie-affected flocks consisted of crossbred animals. In addition, 129 (3.2%) sheep that were part of the case-control analysis of this report carried the VRQ allele which is consistent with the allele frequency reported for other countries with VRQ breeds.

Alternatively, this difference in genotype frequency of scrapie-infected animals between Canada and the UK may be due to the circulation of a different strain(s) of scrapie agent. Transmissible spongiform encephalopathic agents responsible for classical scrapie in small ruminants represent a mosaic of infectious agent harboring distinct biological properties. This is exemplified by experimental studies in which VRQ/VRQ and ARQ/ARQ sheep have been found to be differentially susceptible to 2 sources of scrapie (11). This is also consistent with scrapie outbreaks in other countries (for example, Germany, Spain, Greece) where ARQ/ARQ sheep have demonstrated a similar risk to VRQ carriers or have even been reported as the dominant genotype associated with scrapie in VRQ-encoding breeds (12–14). Indeed, up to 20 different scrapie strains have been identified on the basis of their characteristics in molecular assays and transmission to rodent models (15), some of which were found to attack genotypes differently. Transmission studies to characterize and compare Canadian scrapie strains are in progress.

In Canada, *PRNP* genotyping is currently applied as part of a national strategy that is compulsory for sheep flocks in which classical scrapie has been confirmed. The plan involves whole flock genotyping and the subsequent selective cull of highest risk genotypes. The removal of infected or diseased animals from the national flock produces a consequent reduction of environmental contamination and reduction of the risk of transmission within and between holdings. Similar policies in other countries have demonstrated reductions in the risk of further disease transmission from infected premises (16). The findings of this study support compulsory eradication measures based upon information from codons 136, 154, and 171, as opposed to only codon 171 (QQ) as applied by some jurisdictions. This allows for more economical culling of the highest risk subgroup in Canadian flocks thereby sparing other subgroups that are not at significant risk for scrapie infection. The variability of the sheep *PRNP* gene, however, is greater than that of these standard 3 codons. Recent studies have demonstrated high frequency variants of the ARQ allele with a potentially protective effect to classical scrapie (such as, T_{112}ARQ) (17). Future studies will examine whether such additional polymorphisms can be generalized to Canadian sheep so as to more fully predict their susceptibility to TSE.

Acknowledgments

This project was funded by the Science Division of the Canadian Food Inspection Agency (NTSE-04-04, NTSE-04-CIHR). The authors thank all of the sheep producers who participated in the study and all CFIA field staff involved in the collection and processing of samples.

References

1. Hoinville LJ. A review of the epidemiology of scrapie in sheep. Rev Sci Tech 1996;15:827–852.
2. Annual Scrapie Flock Incidence: 1984–2008, Canadian Food Inspection Agency; [page on the Internet]. Available from: http://www.inspection.gc.ca/english/anima/heasan/disemala/scrtre/incnome.shtml Last accessed May 5, 2010.
3. Eloit M, Adjou K, Coulpier M, et al. BSE agent signatures in a goat. Vet Rec 2005;156:523–524.
4. Goldmann W. PrP genetics in ruminant transmissible spongiform encephalopathies. Vet Res 2008;39:4.

5. Baylis M, Chihota C, Stevenson E, et al. Risk of scrapie in British sheep of different prion protein genotype. J Gen Virol 2004;85:2735–2740.
6. Van Poucke M, Vandesompele J, Mattheeuws M, Van Zeveren A, Peelman LJ. A dual fluorescent multiprobe assay for prion protein genotyping in sheep. BMC Infect Dis 2005;5:13.
7. Bishop G, Farid H. Gene Novas; [page on the Internet]. Available from: https://genenovas.ca/public/stats_pub.html Last accessed May 5, 2010.
8. Sweeney T, Hanrahan JP. The evidence of associations between prion protein genotype and production, reproduction, and health traits in sheep. Vet Res 2008;39:28.
9. Moum T, Olsaker I, Hopp P, et al. Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases. J Gen Virol 2005;86:231–235.
10. Eglin RD, Warner R, Gubbins S, Sivam SK, Dawson M. Frequencies of PrP genotypes in 38 breeds of sheep sampled in the National Scrapie Plan for Great Britain. Vet Rec 2005;156: 433–437.
11. Houston EF, Halliday SI, Jeffrey M, Goldmann W, Hunter N. New Zealand sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1). J Gen Virol 2002;83:1247–1250.
12. Elsen JM, Amigues Y, Schelcher F, et al. Genetic susceptibility and transmission factors in scrapie: Detailed analysis of an epidemic in a closed flock of Romanov. Arch Virol 1999;144:431–445.
13. Acin C, Martin-Burriel I, Goldmann W, et al. Prion protein gene polymorphisms in healthy and scrapie-affected Spanish sheep. J Gen Virol 2004;85:2103–2110.
14. Billinis C, Psychas V, Leontides L, et al. Prion protein gene polymorphisms in healthy and scrapie-affected sheep in Greece. J Gen Virol 2004;85:547–554.
15. Bruce ME, Boyle A, Cousens S, et al. Strain characterization of natural sheep scrapie and comparison with BSE. J Gen Virol 2002;83:695–704.
16. Dawson M, Moore RC, Bishop SC. Progress and limits of PrP gene selection policy. Vet Res 2008;39:25.
17. Laegreid WW, Clawson ML, Heaton MP, Green BT, O'Rourke KI, Knowles DP. Scrapie resistance in ARQ sheep. J Virol 2008;82: 10318–10320.